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An N-Terminal Domain of the Tetracycline Resistance Protein Increases Susceptibility to Aminoglycosides and Complements Potassium Uptake Defects in *Escherichia coli*

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Expression of extrachromosomal *tet* genes increased the susceptibility of gram-negative bacteria to specific aminoglycoside antibiotics. The magnitude of the increase in susceptibility was dependent on the amount and the class of the *tet* gene product (designated Tet) and the bacterial species in which the *tet* gene was expressed. Truncated Tet proteins that contained more than the first 33, but no more than the first 97, N-terminal amino acids of Tet also increased the susceptibility to aminoglycosides and complemented the potassium uptake defects in *Escherichia coli*. The primary structure of this N-terminal Tet fragment has the hydropathic characteristics of a multimeric, transmembrane structure and is highly conserved in three different classes of Tet proteins.

Four classes of plasmid-encoded *tet* genes (A, B, C, and D) in gram-negative bacteria have been described (25). These four classes encode related inner membrane Tet proteins that are distinguished by differences in amino acid sequence and the relative levels of resistance to tetracycline and tetracycline analogs which they confer (18, 23, 26). Expression of *tet* genes has several pleiotropic effects. Tet proteins mediate tetracycline efflux (18, 25), complement certain defects in potassium uptake (10), and confer increased susceptibility to heavy metals (14) and specific organic acids (2, 15, 20). Overexpression of *tet* can also reduce viability (18, 27, 28). We report here that *tet* expression also increases the susceptibility of gram-negative bacteria to specific aminoglycoside antibiotics. Defining the physiological or structural differences between tetracycline-resistant (Tc^r) and tetracycline-sensitive (Tc^s) bacteria that are manifested as a result of these pleiotropic effects could provide new bases for investigating the molecular mechanism of tetracycline efflux and for identifying improved antimicrobial agents.

The physiological basis of the pleiotropic effects of Tet is not known. However, it is reasonable to assume that it is a direct consequence of the structural features of the Tet protein. To study this phenomenon, we have examined the structure-activity relationships of specific fragments of the Tet protein. The results of these studies indicate that truncated Tet proteins containing as little as the first 97 N-terminal amino acids (Tet_{97}), although incapable of conferring Tc^r, increased the susceptibility to aminoglycosides and complemented potassium uptake defects in *Escherichia coli*. Tet_{97} has the characteristics of a multimeric transmembrane protein, and its amino acid sequence may be related to those of other membrane proteins, altered forms of which also affect susceptibility to aminoglycosides.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in these experiments are listed in Table 1. Tc^r strains were derived from Tc^s strains by calcium chloride-mediated plasmid transformation (21) or transposition after infection with defective lambda bacteriophage (KN561) carrying the transposon Tn*10* (9).

Construction of plasmids. Plasmid pCC42, a derivative of pBR322 with an inducible *tet* gene, was constructed by ligating the 1,844-nucleotide *Pvu*II-*Bam*HI fragment of pSC101 that contains the *tetR* transcription repressor gene (1) to the 3,988-nucleotide *Eco*RI-*Bam*HI fragment of pBR322 that contains the *tet* and *bla* structural genes and the ColE1 origin of plasmid DNA replication (32). The regulation of the pCC42 *tet* structural gene by the *tetR* repressor was verified by comparing the growth rates of HB101, HB101(pBR322), and HB101(pCC42) in L broth containing 20 μg of tetracycline per ml, a concentration that is inhibitory for Tc^s HB101, with and without prior derepression (induction) of the *tetR* repressor with 0.5 μg of acid-inactivated tetracycline per ml. Acid-inactivated tetracycline was prepared by refluxing tetracycline in 0.2 N H_2SO_4 for 20 h. The pH was then adjusted to 6.0 with NaOH, and contaminating 5a,6-anhydrotetracycline was removed by precipitation in the cold. Chromatographic analysis on octadecylsilane columns with 30% methanol–70% 1 mM EDTA indicated that the final product contained less than 1.0% unreacted tetracycline. Accordingly, acid-inactivated tetracycline prepared in this manner had no antibacterial activity at 150 μg/ml against *E. coli*. Acid-inactivated tetracycline was approximately 10-fold more effective than tetracycline at inducing *tet* gene expression (data not shown).

Plasmid pCC100 was recovered from mutagenized HB101(pBR322) by selecting for Tc^r at 75 μg/ml. Preliminary data indicate that the segment of the pCC100 plasmid that is responsible for the increased Tc^r is not located within the *tet* gene itself, but lies within the *Pst*I-*Pvu*II fragment that contains the origin of replication. This suggests that the mutation may affect the plasmid copy number.

Mutant pBR322 plasmids lacking specific portions of the *tet* structural gene were constructed by deleting DNA between the pairs of restriction sites shown in Fig. 2. Briefly, plasmid DNA was digested with the specified pairs of restriction enzymes; if necessary, the staggered ends were

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TABLE 1. Strains and plasmids

Strain or plasmid	Phenotype[a]	Structure	Source or reference
Strains			
E. coli K-12 HB101	RecA$^-$ Sm^{cr} Tc^s		4
E. coli K-12 LE392	RecA$^+$ Sm^s Tc^s		22
E. coli K-12 TK2205	Tc^s Kdp$^-$ TrK$^-$		10
E. coli K-1 LA396	Tc^s		H. Bially
S. typhimurium LT2-2	Tc^s		9
Plasmids			
Class A			
pJOE105	Ap^{cr} Tc^{ir}		31
Class B			
Tn*10*	Tc^{ir}		9
Class C			
pBR322	Ap^{cr} Tc^{cr}		3
pCC99	Ap^s Tc^{cr}	Δ*Pst*I-*Eco*RI	This study
pCC47	Ap^{cr} Tc^s	Δ*Eco*RI-*Nru*I	This study
pCC42	Ap^{cr} Tc^{ir}	+*tetR* at *Eco*RI	This study
pCC100	Ap^{cr} high Tc^{cr}		This study
pSC101	Tc^{ir}		7
pACYC184	Cm^{cr} Tc^{cr}		6
pACYC184*tet*56	Cm^{cr} Tc^s	aa 25–68	This study
pACYC184*tet*62	Cm^{cr} Tc^s	aa 25–68	This study
pACYC184*tet*51	Cm^{cr} Tc^s	aa 158–215	This study
pACYC184*tet*53	Cm^{cr} Tc^s	aa 215–298	This study
pACYC184*tet*60	Cm^{cr} Tc^s	aa 215–298	This study
pCC44	Ap^{cr} Tc^s	Δ*Eco*RV-*Pvu*II	This study
pCC52	Ap^{cr} Tc^s	Δ*Bam*HI-*Pvu*II	This study
pCC56	Ap^{cr} Tc^s	Δ*Sal*I-*Pvu*II	This study
pCC58	Ap^{cr} Tc^s	Δ*Nru*I-*Pvu*II	This study
pCC43	Ap^{cr} Tc^s	Δ*Eco*RV-*Nru*I	This study
pCC45	Ap^{cr} Tc^s	Δ*Bam*HI (S1 digested)	This study
pCC1-1	Ap^{cr} Tc^s	+ 30 nt at *Bam*HI	This study
pCC1-2	Ap^{cr} Tc^s	+120 nt at *Bam*HI	This study
pCC1-3	Ap^{cr} Tc^s	+220 nt at *Bam*HI	This study
pCC1-4	Ap^{cr} Tc^s	+880 nt at *Bam*HI	This study
Class D			
pSL101	Tc^{ir}		23

[a] Only phenotypes that are relevant to this study are indicated. Abbreviations: ir, inducible resistance; cr, constitutive resistance; aa, amino acid; nt, nucleotide. The location of each pACYC184 *tet* point mutation was mapped as described in Materials and Methods. The segment of the pACYC184 Tet amino acid sequence inferred to contain the mutation is indicated. For pBR322 *tet* mutations, Δ indicates that DNA has been deleted between the specified restriction sites and + indicates that DNA has been inserted into the specified restriction site. The LD_{90}s of tetracycline for all Tc^r and Tc^s strains were greater than 60 μg/ml and less than 2.5 μg/ml, respectively.

made flush with S1 nuclease, and the DNA was then recircularized with T4 DNA ligase (22). The Tet amino acid sequence encoded by the DNA downstream of the pBR322 *Bam*HI site was also changed by two additional methods. First, plasmid pBR322 DNA was linearized with *Bam*HI, the staggered ends were made flush with S1 nuclease, and the DNA was then recircularized with T4 DNA ligase (22). Second, random human *Sau*3A DNA fragments were ligated into the *Bam*HI site (see Fig. 2). All modifications completely abolished Tc^r (Table 1).

Point mutations in the *tet* gene were derived and mapped as follows. Plasmid pACYC184 DNA was mutagenized in vitro with hydroxylamine (9) and introduced into calcium chloride-treated *E. coli* HB101 (21). Transformants harboring mutant *tet* genes were selected by resistance to chloramphenicol and screening for Tc^s. The location of each *tet* point mutation was then determined by deletion mapping. Briefly, each mutant was introduced into a series of *E. coli* strains that contained pBR322 plasmids from which the promoter and defined amounts of downstream DNA had been deleted. The presence or absence of Tc^r recombinants arising from recombination between the *tet* mutation on plasmid pACYC184 and each deletion mutation on plasmid pBR322 was then determined.

Measurement of antibiotic resistance. The antibiotic concentration that reduced bacterial plating efficiency by 90% (the LD_{90}) was determined as follows. Overnight cultures were diluted with fresh L broth (with or without 0.5 μg of acid-inactivated tetracycline per ml) to an A_{600} of approximately 0.05 and incubated at 37°C with vigorous agitation until the A_{600} reached 0.5. The log-phase cultures were then diluted serially with L broth, and Tc^s or Tc^r cells (2×10^3 to 5×10^3) were spread on paired L-broth agar plates containing the specified concentrations of the indicated antibiotics (Sigma Chemical Co.) with and without 0.5 μg of acid-inactivated tetracycline per ml. In most experiments, susceptibility was measured at increments of antibiotic concentration approximately equal to 10% of the LD_{90} of the Tc^s strain established in preliminary studies. Plates were incubated at 37°C for 18 to 24 h, after which the number of colonies was determined.

Complementation of potassium uptake defects. Plasmids were introduced into calcium chloride-treated *E. coli* TK2205 cells as described previously (21). Each strain was

TABLE 2. Km^s of Tc^s and Tc^r strains

Strain	Kanamycin LD_{90} (μg/ml) (SEM)[a]
HB101	0.82 (0.07)
HB101(pBR322)	0.51 (0.06)
LE392	0.29 (0.02)
LE392(pBR322)	0.15 (0.01)
LA396	1.16 (0.05)
LA396(pBR322)	0.78 (0.03)
LT2	3.1 (0.27)
LT2(pBR322)	0.93 (0.12)

[a] The LD_{90}s of kanamycin for the Tc^s and Tc^r strains were measured in three independent experiments as described in Materials and Methods. The mean LD_{90}s and the standard errors of the means (SEM) are presented.

grown overnight in minimal medium containing 0.2% glucose and 115 mM potassium ion at 37°C (10). Sufficient cells to produce an A_{600} in 50 ml were concentrated by centrifugation, and the medium was decanted. The cells were then suspended in 50 ml of prewarmed minimal medium containing 0.2% glucose and either 0, 2.5, or 115 mM potassium ion (K_0, $K_{2.5}$, and K_{115}, respectively) and shaken for 60 to 90 min at 37°C to reequilibrate the cellular potassium pool. The doubling times of the cultures were then determined at 37°C from the subsequent increase in the A_{600}.

RESULTS

Increased susceptibility of Tc^r bacteria to aminoglycosides. The presence of plasmid pBR322 in each of three *E. coli* strains and a *Salmonella typhimurium* strain significantly increased the susceptibilities of these strains to kanamycin (Table 2). The mean LD_{90}s of kanamycin for the three Tc^r *E. coli* strains varied between 52 and 68% of that for the isogenic Tc^s controls. Chromosomal mutations conferring $RecA^-$ and streptomycin-resistant (Sm^r) phenotypes did not significantly affect the plasmid-dependent increase in Km^s. The average effect of plasmid on Km^s was nearly twofold higher in the *S. typhimurium* strain than in the *E. coli* strains. Expression of the pBR322 *tet* gene also reduced comparably the LD_{90}s of three closely related aminoglycosides: gentamicin, amikacin, and tobramycin. However, no measurable effect was noted on the susceptibility to several other inhibitors, including another aminoglycoside, kasugamycin, and the aminocyclitol spectinomycin (data not shown).

***tet* expression required for increased aminoglycoside susceptibility.** The plasmid-dependent increase in Km^s was not suppressed by deletion of DNA containing the plasmid pBR322 *bla* gene promoter (plasmid pCC99), but was completely suppressed by deletion of DNA containing the pBR322 *tet* gene promoter (plasmid pCC47) (Table 3). To confirm that the increased susceptibility to aminoglycosides resulted from the expression of the *tet* gene, we measured Km^s in strains in which *tet* expression is inducible. Plasmid pCC42 expressed the *tet* gene only when derepressed with tetracycline or a tetracycline analog such as acid-inactivated tetracycline (see Materials and Methods), but was otherwise identical to pBR322 which expresses the *tet* gene constitutively (22). The effect of inducers of *tet* gene expression on Km^s was compared in HB101, HB101(pBR322), and HB101(pCC42). The LD_{90}s of kanamycin for HB101 and HB101(pCC42) were identical when assayed without acid-inactivated tetracyline (Table 3). However, in the presence of acid-inactivated tetracycline, the LD_{90} of kanamycin for HB101(pCC42) fell to a value that was indistinguishable from that of kanamycin for HB101(pBR322). Acid-inactivated tetracycline did not significantly affect the Km^s of either HB101 or HB101(pBR322) (Table 3).

The effect of *tet* expression on Km^s was also reproduced with two unrelated plasmids that contain the same class C *tet* gene as pBR322. The *tet* gene of plasmid pACYC184, like that of pBR322, is expressed constitutively (6). HB101 (pACYC184) was more Km^s than plasmid-free HB101 was in the absence of acid-inactivated tetracycline (Table 3). The *tet* gene of plasmid pSC101, like that of pCC42, is normally repressed (7). Accordingly, HB101(pSC101) and plasmid-free HB101 were equally Km^s in the absence of acid-inactivated tetracycline, but HB101(pSC101) became more Km^s in its presence (Table 3). Together, these results indicate that the increased susceptibility to aminoglycosides requires *tet* gene expression.

Correlation of increased susceptibility to aminoglycosides with the level of *tet* gene expression. Although plasmids pSC101, pACYC184, and pBR322 contain the same *tet* gene (3, 6, 7), they differ in copy number (22), resulting in different *tet* gene dosages. pSC101 has a lower copy number than either pACYC184 or pBR322. The copy number of the mutant pBR322 plasmid pCC100 has not been determined. However, the fact that the mutation is not in the *tet* gene is consistent with the possibility that the mutation increases plasmid copy number (see Materials and Methods). HB101(pBR322) and HB101(pACYC184) both expressed greater Tc^r and greater Km^s than acid-inactivated tetracycline-induced HB101(pSC101) (Table 3). HB101(pCC100) expressed greater Tc^r and greater Km^s than HB101 (pBR322), HB101(pACYC184), or acid-inactivated tetracycline-induced HB101(pSC101). Linear regression analysis indicated a highly significant correlation between the level of

TABLE 3. Km^s and Tc^s of Tc^r strains

Strain	ATc[a] added	LD_{90} (μg/ml) (SEM) of[b]:	
		Kanamycin	Tetracycline
HB101	−	0.94 (0.09)	2 (0.1)
HB101	+	0.80 (0.08)	
HB101(pBR322)	−	0.54 (0.06)	65 (3.9)
HB101(pBR322)	+	0.45 (0.05)	
HB101(pCC99)	−	0.45	
HB101(pCC47)	−	0.95	2
HB101(pCC42)	−	0.93 (0.03)	
HB101(pCC42)	+	0.53 (0.03)	
HB101(pSC101)	−	0.90	40 (5.7)
HB101(pSC101)	+	0.63 (0.04)	
HB101(pACYC184)	−	0.47 (0.10)	48 (7.5)
HB101(pCC100)	−	0.31 (0.06)	115 (6.7)
HB101(pJOE105)	−	0.87 (0.07)	240 (6.8)
HB101(pJOE105)	+	0.63 (0.04)	
HB101::Tn*10*	−	2.06 (0.06)	198 (2.5)
HB101::Tn*10*	+	1.44 (0.07)	
HB101(pSL101)	−	0.88 (0.08)	177
HB101(pSL101)	+	0.70 (0.03)	
LT2	−	3.10 (0.27)	3
LT2(pBR322)	−	0.93 (0.12)	88 (4.4)

[a] ATc, Acid-inactivated tetracycline. Symbols: −, absent; +, present at 0.5 μg/ml.

[b] The mean LD_{90}s of kanamycin (with or without acid-inactivated tetracycline) and tetracycline were measured for each strain as described in Materials and Methods. The standard errors of the means (SEM) are given in parentheses.

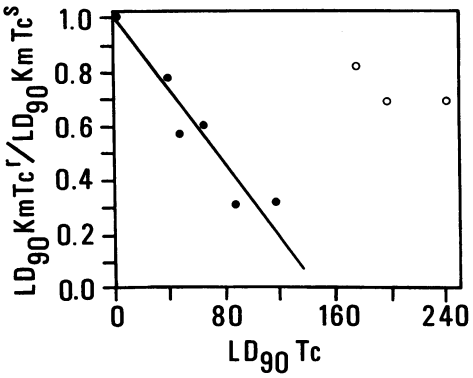

FIG. 1. Relationship between levels of Tc^r and increased Km^s. The data in Table 3 were used to calculate the fractional reduction in the mean LD_{90} of kanamycin that results from *tet* expression in each strain (LD_{90} Km Tc^r/LD_{90} Km Tc^s). These values were then plotted versus the mean LD_{90} of tetracycline for the strain. The data for strains containing class C *tet* determinants (●) were fitted to a linear plot by the least-squares equation. The linear regression coefficient (−0.952) indicates that there is a very strong linear correlation between these two parameters. The data for the strains containing the class A, B, and D *tet* genes (○) are shown for comparison.

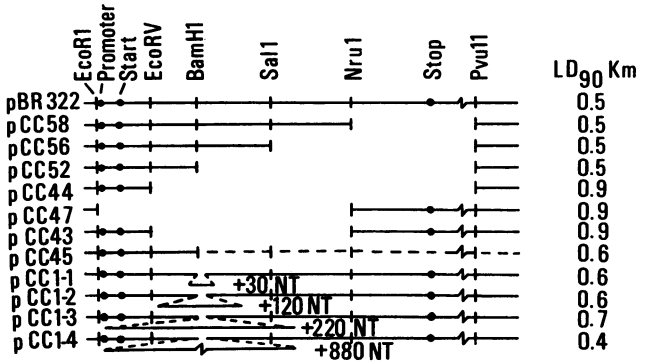

FIG. 2. Mapping of the Tet domain responsible for increased susceptibility to aminoglycosides. Mutant pBR322 plasmids were constructed by deleting or inserting DNA at the indicated restriction sites as described in Materials and Methods. Deleted DNA is indicated by gaps in the solid line, putative changes in the reading frame are indicated by a dashed line, and insertions of the specified lengths are flanked by dashed lines. The relevant restriction sites, the promoter, and the translation start and stop codons are also shown. The LD_{90} of kanamycin for each plasmid-containing strain and plasmid-free HB101 was compared in three independent experiments as described in Materials and Methods. Data from a single experiment are shown. Two independent isolates of most deletion plasmids were tested and yielded similiar results. The LD_{90}s for HB101 containing 10 different human DNA insertion plasmids were examined. Of the 10, 9 were more Km^s than plasmid-free HB101 was. Abbreviations: Km, kanamycin; NT, nucleotide.

Tc^r and the magnitude of the increase in Km^s in *E. coli* and *S. typhimurium* strains carrying class C *tet* genes (Fig. 1).

Increased Km^s conferred by all classes of *tet* genes. The finding that the class C *tet* gene conferred increased Km^s is of greater significance if the increased susceptibility is also conferred by the other classes of *tet* gene determinants commonly encountered in gram-negative organisms (18, 23, 25, 26). To address this question, we compared Km^s with and without acid-inactivated tetracycline in HB101 containing inducible members of the other three classes (i.e., A, B, and D) of *tet* determinants. The mean LD_{90}s of kanamycin for strains containing class A, B, and D determinants in the presence of acid-inactivated tetracycline were 72%, 70% and 80%, respectively, of the LD_{90} of the matched controls in the absence of acid-inactivated tetracycline (Table 3). The quantitative relationship between the level of Tc^r and the magnitude of the increase in Km^s was different in these strains than in strains containing the class C *tet* gene (Fig. 1). The LD_{90} of kanamycin in the absence of acid-inactivated tetracycline was greater in the strain containing Tn*10* than in plasmid-free strains. The basis of this difference is not known.

Mapping the Tet domain that confers the pleiotropic effects of Tet. To determine whether the increased Km^s of Tc^r bacteria required the activity of a functional Tet protein, we first assessed the effect on Km^s of defined structural *tet* gene mutations that abolished Tc^r. None of six regionally mapped *tet* point mutations that abolished Tc^r suppressed the *tet*-dependent increase in Km^s (not shown). Therefore, the *tet*-dependent increase in Km^s does not require a functional Tet protein.

These results suggested that plasmids lacking specific portions of the *tet* gene might be used to map the minimum Tet domain that is necessary to express the pleiotropic effects of Tet. Mutant pBR322 plasmids were constructed by deleting the portions of the *tet* gene diagrammed in Fig. 2. Although every deletion abolished Tc^r (Table 1), only deletions that removed the *tet* gene DNA sequence upstream of the *Bam*HI restriction site suppressed the increased Km^s (Fig. 2). All plasmids that contained the *tet* gene DNA between the *Eco*RI and *Bam*HI restriction sites conferred increased Km^s. Likewise, insertion of random 30- to 880-nucleotide *Sau*3A fragments of human DNA into the *tet* gene *Bam*HI site (e.g., pCC1-1) abolished Tc^r (Table 1), but did not suppress Km^s (Fig. 2). Changes in the reading frame of mRNA encoded by DNA downstream of the *Bam*HI site (e.g., pCC45) also abolished Tc^r (Table 1), but did not suppress the increased Km^s (Fig. 2). Thus, the increase in Km^s that accompanies *tet* expression requires neither the activity of a functional Tet protein nor a structurally intact Tet protein.

Mutations (*kdpABC*, *trkA*, and *trkD*) in *E. coli* TK2205 significantly impair potassium uptake (10, 13). Accordingly, TK2205 cannot grow without a high external concentration of potassium ions. This defect is partially complemented by expression of the pBR322 *tet* gene (10). The doubling time of

TABLE 4. Effect of *tet* gene mutations on the rate of growth in potassium-poor medium

Strain[a]	Doubling time (min) (SEM) at[b]:		
	K_0	$K_{2.5}$	K_{115}
TK2205	NG[c]	NG	56 (0.48)
TK2205(pBR322)	NG	63 (0.89)	56 (0.50)
TK2205(pCC42)			
−ATc	NG	300	57
+ATc	NG	72	60
TK2205(pCC44)	NG	267 (31.8)	57 (0.66)
TK2205(pCC45)	NG	58	56
TK2205(pCC52)	NG	101 (11.8)	57 (0.66)
TK2205(pCC57)	NG	60	60
TK2205(pCC59)	NG	75	55

[a] ATc, Acid-inactivated tetracycline.

[b] The mean growth rates of each strain were measured in minimal medium containing 0, 2.5, and 115 mM potassium ion (K_0, $K_{2.5}$, and K_{115}, respectively) as described in Materials and Methods. Standard errors of the means (SEM) are shown in parentheses.

[c] NG, No growth.

TK2205(pBR322) in medium containing only 2.5 mM potassium ion ($K_{2.5}$) was approximately 110% of the doubling time of plasmid-free TK2205 in medium containing 115 mM potassium ion (K_{115}) (Table 4). Complementation of the potassium uptake defect requires *tet* gene expression; TK2205 containing plasmid pCC42 will not grow in $K_{2.5}$ unless *tet* gene expression is induced by tetracycline or acid-inactivated tetracycline.

We next determined whether the N-terminal portion of the Tet protein also was sufficient to complement the potassium uptake defect. A summary of these experiments is presented in Table 4. All strains failed to grow in potassium-free medium (K_0) and had doubling times of 55 to 60 min in K_{115}. There was complete concordance between the ability of the mutant plasmids to increase Km^s (Fig. 2) and to grow in $K_{2.5}$ (Table 4). The Tet protein initiation codon, the *Eco*RV site, and the *Bam*HI site are located at nucleotides 86, 185, and 375, respectively (Fig. 2). Thus, a fragment containing more than the first 33 amino acid residues, but no more than the first 97 amino acid residues, of the class C Tet protein (Tet_{97}) was sufficient to confer at least two of the pleiotropic effects of native TET, but not to confer Tc^r. The fact that these pleiotropic effects were not suppressed by several different *tet* mutations that altered the amino acid sequence encoded by DNA downstream from the *Bam*HI site indicates that the expression of these pleiotropic effects is independent of the C-terminal amino acid sequence.

DISCUSSION

Factors affecting Tet-dependent aminoglycoside susceptibility. Each of the strains examined became more Km^s when the *tet* genes were expressed. A direct relationship existed between the levels of Tc^r and the increase in Km^s in strains containing the same class C *tet* gene (Fig. 1). The magnitude of the increase in Km^s was also affected by the bacterial host in which the *tet* gene was expressed. For example, pBR322 reduced the LD_{90} of kanamycin an average of 40% in three different *E. coli* strains, but 69% in the *S. typhimurium* strain (Table 2). Nonetheless, the quantitative relationship between the levels of Tc^r and Km^s in these strains was similar (Fig. 1). Thus, the difference in Km^s in these species may reflect strain-specific factors that affect the level of *tet* gene expression. Increasing the strength of the pBR322 *tet* gene promoter also increases Tc^r and susceptibility to fusaric acid (15), whereas decreasing the strength of the pBR322 *tet* gene promoter decreases Tc^r and the ability to complement potassium uptake defects (10). Thus, the magnitude of at least three of the known pleiotropic effects of *tet* is directly related to the level of *tet* gene expression, as defined by the level of Tc^r.

Another variable that affects Km^s is the class of Tet protein that is expressed. Members of all four Tet classes increased Km^s in HB101 (Table 3). However, the quantitative relationship between Tc^r and Km^s expressed by the class A, B, and D *tet* genes was different from that expressed by the class C *tet* gene (Fig. 1). Heterogeneity in the effects of the individual *tet* classes on resistance to tetracycline and its analogs and complementation of potassium uptake defects also have been reported (10, 18, 26).

Correlating the functional differences between the individual classes of Tet proteins with the known differences in their amino acid sequences could help to define the structural basis of the pleiotropic effects of Tet. Increased Km^s is conferred by class A to D Tet proteins and by fragments of the class C Tet protein containing Tet_{97}. We compared the amino acid sequences of Tet_{97} among the three classes of Tet proteins for which sequence data are available (16, 29, 32, 34). Two regions of TET_{97} differ significantly in the conservation of their amino acid sequences. Between pBR322 Tet residues 1 and 15, only 2 of the 15 residues (13%) are identical in the three Tet proteins. A more highly conserved region, however, is located between pBR322 Tet residues 16 and 97, in which 49 of the 82 residues (60%) are identical in the three Tet proteins. Hydropathic analyses performed by using the algorithims of Kyte and Doolittle (17) and Eisenberg et al. (11) also identified three potential multimeric, membrane-spanning structures in Tet_{97} such as those found in multiprotein complexes and membrane channel-forming proteins (11). These characteristics were conserved among the three classes of Tet proteins. It is reasonable to assume that the amino acid residues and/or the structural motifs which they dictate and that are essential for the expression of the pleiotropic effects of Tet are located within the more highly conserved region.

Basis of the pleiotropy of *tet* expression. We have shown that two of the pleiotropic effects of Tet, increasing susceptibility to aminoglycosides and complementing potassium uptake defects, are conferred by Tet_{97}. Other data suggest that Tet_{97} also increases susceptibility to fusaric acid and cadmium (our unpublished studies). Similiarly, Moyed et al. have reported that all mutations that suppress the effect of Tet overexpression on viability map to a restriction fragment encoding the first 100 N-terminal Tet amino acids (27, 28). Since many of these mutations did not affect Tc^r, it is more likely that they affected the Tet protein than the *tet* promoter or the *tet* mRNA ribosome-binding site (27, 28). Together, these data suggest that all of the pleiotropic effects of Tet are mediated by Tet_{97} and therefore may have a common mechanistic basis.

Expression of either native class C Tet or Tet_{97} increases potassium uptake (10). This suggests that a common basis for the increased susceptibilities of Tc^r bacteria to aminoglycosides, cadmium, and fusaric acid might also be increased uptake of these compounds. One possibility that could account for the increased uptake of these compounds is that integration of Tet into the membrane results in a nonspecific increase in permeability. However, as described above, most antibiotics that we and others have examined have equal potency against Tc^s and Tc^r strains (2; our unpublished results). Moreover, *tet* expression increases susceptibility only to specific aminoglycosides (e.g., kanamycin but not kasugamycin) and to specific organic acids (2). Therefore, the effect of *tet* expression is not nonspecific.

Another possibility is that the uptake of these compounds is directly mediated by Tet. For example, the uptake of these compounds could be directly coupled to the efflux of tetracycline. This explanation can be excluded because the pleiotropic effects of Tet are conferred in the absence of tetracycline by constitutively expressed *tet* genes (Tables 2 and 3) (10, 14, 15, 20). Similarly, Dosch et al. have suggested the Tet protein could have a low substrate specificity and a K_m for influx that favors the uptake of these specific molecules (10). In other words, the increased uptake of compounds such as kanamycin could occur by the reverse of the same mechanism which mediates tetracycline efflux. This possibility seems unlikely, since the pleiotropic effects of Tet are conferred by severely truncated Tet proteins which are unable to confer Tc^r (Fig. 2).

The mechanism which we propose is that Tet increases the transmembrane proton gradient (Δp), possibly via an effect

on membrane respiratory proteins or ATP synthetase. This proposal is based on the following considerations. First, the uptake of most, if not all, of the compounds known to be affected by *tet* expression is also affected by Δp. The uptake of aminoglycosides in gram-positive and gram-negative bacteria (5, 8, 12, 24), cadmium in *Staphylococcus aureus* (33), and potassium in the *E. coli* TK2205 mutant (13) and the Tet-dependent efflux of tetracycline in *E. coli* (18) are all dependent on the membrane potential. The mode of fusaric acid uptake is not known. However, lipophilic acids similiar to fusaric acid have been used to measure the transmembrane pH gradient in *E. coli*, and the accumulation of these organic acids is proportional to the magnitude of the pH gradient (30). Therefore, Dosch et al. have interpreted the fact that the antibacterial activity of fusaric acid is greatest at low pH (2) to indicate that the uptake of fusaric acid may be dependent on the transmembrane pH gradient (10).

The second line of evidence supporting our hypothesis is that mutations that reduce the levels of functional cytochromes and coenzyme Q also reduce the membrane potential, the uptake of aminoglycosides, and bacterial susceptibility to aminoglycosides (5, 8). Likewise, inhibitors and mutations that mimic the proposed action of the Tet protein, i.e., that affect the ATP synthetase complex so as to reduce proton influx, also increase the membrane potential, the uptake of aminoglycosides, and bacterial susceptibility to aminoglycosides (5, 8).

The third line of evidence is that the amino acid sequence of Tet_{97} is similiar to the sequences of membrane proteins involved in respiration, altered forms of which can also affect susceptibility to aminoglycosides (5, 8). Using the FASTP and RDF algorithims (19), we compared the amino acid sequence of the N-terminal 97 residues of the class C Tet protein with the sequences of the 4,253 other proteins in the National Biomedical Research Foundation database. Similiarities to the subunits of multimeric respiratory proteins, including cytochrome *b* (*Z* score of 4.5 standard deviations) were detected. One possibility suggested by these similiarities to respiratory proteins is that Tet_{97} possesses structural features that enable it to increase Δp, perhaps by directly affecting the activities of protein complexes involved in energy transduction or proton transport or both.

An observation that is apparently inconsistent with the hypothesis that the pleiotropic effects of Tet are caused by an increase in Δp is that expression of the class B Tn*10* *tet* gene does not measurably increase the growth rate of TK2205 in potassium-poor medium (10). However, the effect of the class B Tet protein on potassium uptake may simply be smaller than that of class C Tet and may therefore not be detected by observing changes in growth rate.

Our hypothesis makes several testable predictions. Calculating from the data of Damper and Epstein, we would expect the membrane potentials of HB101 and HB101 (pBR322) to differ by about 10 mV. The hypothesis also predicts that *tet* expression will increase the pH gradient and Δp-dependent aminoglycoside uptake. We would also expect that mutations affecting ATP synthetase or other respiratory proteins might suppress the pleiotropic effects of Tet. Likewise, it may also be possible to isolate chromosomal mutations in other genes that suppress specifically the pleiotropic effects of Tet. These studies may define genes whose products interact with Tet in the membrane to cause its pleiotropic effects. Together, such approaches will further elucidate the physiological and structural basis of the pleiotropic effects of Tet.

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LITERATURE CITED

1. **Bernardi, A., and F. Bernardi.** 1985. Complete sequence of pSC101. Nucleic Acids Res. **12**:9415–9426.
2. **Bochner, B., H. Huang, G. Schiever, and B. Ames.** 1980. Positive selection for loss of tetracycline resistance. J. Bacteriol. **143**: 926–933.
3. **Bolivar, F., R. L. Rodriques, P. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95–113.
4. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459–472.
5. **Bryan, L. E., and H. E. Van Den Elzen.** 1977. Effects of membrane energy mutation and cations on streptomycin and gentamicin accumulation by bacteria: a model for entry of streptomycin and gentamicin in susceptible and resistant bacteria. Antimicrob. Agents Chemother. **12**:163–177.
6. **Chang, A. C. Y., and S. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. J. Bacteriol. **134**: 1141–1156.
7. **Cohen, S. N., and A. C. Y. Chang.** 1977. Revised interpretation of the origin of the pSC101 plasmid. J. Bacteriol. **132**:734–737.
8. **Damper, P., and W. Epstein.** 1981. Role of membrane potential in bacterial resistance to aminoglycoside antibiotics. Antimicrob. Agents Chemother. **20**:803–808.
9. **Davis. R. W., D. Botstein, and J. R. Roth (ed.).** 1980. Advanced bacterial genetics, p. 94–97. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
10. **Dosch, D., F. Salvacion, and W. Epstein.** 1984. Tetracycline resistance element of pBR322 mediates potassium transport. J. Bacteriol. **160**:1188–1190.
11. **Eisenberg, D., E. Schwarz, M. Komaromy, and R. Wall.** 1984. Analysis of membrane and surface protein sequence with the hydrophobic moment plot. J. Mol. Biol. **179**:125–142.
12. **Eisenberg, E., L. Mandel, R. Kaback, and M. Miller.** 1984. Quantitative association between electrical potential across the cytoplasmic membrane and early gentamicin uptake and killing in *Staphylococcus aureus*. J. Bacteriol. **157**:863–867.
13. **Epstein, W., and L. Laimins.** 1980. Potassium transport in *E. coli*: diverse systems with common control by osmotic forces. Trends Biochem. Sci. **5**:21–23.
14. **Griffith, J. K., J. M. Buckingham, J. L. Hanners, C. E. Hildebrand, and R. A. Walters.** 1982. Plasmid-conferred tetracycline resistance confers collateral cadmium sensitivity to *E. coli* cells. Plasmid **8**:86–88.
15. **Herrin, G., D. Russel, and G. Bennet.** 1982. A stable derivative of pBR322 conferring increased tetracycline resistance and increased sensitivity to fusaric acid. Plasmid **7**:290–293.
16. **Hillen, W., and K. Schollmeier.** 1983. Nucleotide sequence of the Tn*10* encoded tetracycline resistance gene. Nucleic Acids Res. **11**:525–539.
17. **Kyte, J., and R. Doolittle.** 1982. A simple method for displaying the hydropathic characteristics of a protein. J. Mol. Biol. **157**: 105–132.
18. **Levy, S.** 1984. Resistance of the tetracyclines, p. 191–240. *In* L. E. Bryan (ed.), Antimicrobial drug resistance. Academic Press, Inc., New York.
19. **Lipman, D., and W. Pearson.** 1985. Rapid and sensitive protein similiarity searches. Science **227**:1435–1441.
20. **Malloy, S., and W. Nunn.** 1981. Selection for loss of tetracycline resistance by *Escherichia coli*. J. Bacteriol. **145**:1110–1112.

21. **Mandel, M., and A. Higa.** 1970. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. **53**:159–162.
22. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Marshall, B., C. Tachibana, and S. B. Levy.** 1983. Frequency of tetracycline resistance determinant classes among lactose-fermenting coliforms. Antimicrob. Agents Chemother. **24**:835–840.
24. **Mates, S., E. Eisenberg, L. Mandel, L. Patel, R. Kaback, and M. Miller.** 1982. Membrane potential and gentamicin uptake in *Staphylococcus aureus*. Proc. Natl. Acad. Sci. USA **79**:6693–6697.
25. **McMurry, L., R. Petrucci, and S. B. Levy.** 1980. Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **77**:3974–3977.
26. **Mendez, B., C. Tachibana, and S. B. Levy.** 1980. Heterogeneity of tetracycline resistance determinants. Plasmid **3**:99–108.
27. **Moyed, H. S. and K. P. Bertrand.** 1983. Mutations in multicopy Tn*10* *tet* plasmids that confer resistance to inhibitory effects of inducers of *tet* gene expression. J. Bacteriol. **155**:557–564.
28. **Moyed, H. S., T. T. Nguyen, and K. P. Bertrand.** 1983. Multicopy Tn*10* *tet* plasmids confer sensitivity to induction of *tet* gene expression. J. Bacteriol. **155**:549–556.
29. **Peden, K. W.** 1983. Revised sequence of the tetracycline resistance gene of pBR322. Gene **22**:277–280.
30. **Ramos, S., S. Schuldiner, and H. Kaback.** 1976. The electrochemical gradient of protons and its relationship to active transport in *E. coli* membrane vesicles. Proc. Natl. Acad. Sci. USA **73**:1892–1896.
31. **Schoffl, R., W. Arnold, A. Puhler, J. Altenbuchner, and R. Schmitt.** 1981. The tetracycline-resistant transposons Tn*1721* and Tn*1771* have three 38-base-pair repeats and generate five-base-pair direct repeats. Mol. Gen. Genet. **181**:87–94.
32. **Sutcliffe, G.** 1979. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. **43**:77–90.
33. **Tynecka, Z., Z. Gos, and J. Zajac.** 1981. Energy-dependent efflux of cadmium coded by a plasmid resistance determinant in *Staphylococcus aureus*. J. Bacteriol. **147**:313–319.
34. **Waters, S. H., P. Rogowsky, J. Grinstead, J. Altenbuchner, and R. Schmitt.** 1983. The tetracycline resistance determinants of RP1 and Tn*1721*: nucleotide sequence analysis. Nucleic Acids Res. **11**:6089–6104.